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EVIDENCE FOR THE INVOLVEMENT OF SINGLET OXYGEN IN THE PHOTODESTRUCTION BY CHLOROALUMINUM PHTHALOCYANINE TETRASULFONATE

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In recent years, chloroaluminum phthalocyanine tetrasulfonate (AlPCTS) has been shown to be a promising photosensitizer for the photodynamic therapy (PDT) of cancer. Although its mechanism of photodynamic action is not well defined, AlPCTS is going to be under clinical trials of PDT. In this study, *in vitro* addition of AlPCTS to a suspension of rat epidermal microsomes followed by irradiation with red light (~675 nm) resulted in significant destruction of cytochrome P-450 and associated monooxygenase activities. The photodestructive effect was dependent on both the dose of AlPCTS and the duration of light exposure. Studies using various quenchers of reactive oxygen species showed that only scavengers of singlet oxygen such as histidine, 2,5-dimethylfuran, β-carotene and sodium azide afforded substantial protection against photodestruction. Our data indicate the direct involvement of singlet oxygen in the AlPCTS-mediated photodestructive process.

The use of photosensitizing chemicals combined with light is known as photodynamic therapy (PDT) and affords the potential for targeted destruction of malignant neoplasms (1). Porphyrin derivatives, the major class of chemicals that have been studied in this regard (2,3), unfortunately possess several intrinsic disadvantages (4,5). This has led to the search for new photosensitizers with an improved therapeutic ratio. Chloroaluminum phthalocyanine tetrasulfonate (AlPCTS) has shown promise in this regard. It is non-toxic even at high doses in several mammalian species (6-9), and when injected to animals, has a relatively short retention time in tissues including normal skin (5,10). AlPCTS has been shown to preferentially localize in tumor tissues and on exposure to red light (600-700 nm) results in tissue necrosis (7,8,11-13). Clinical trials using AlPCTS in PDT are about to start in Israel (14), however the exact mechanism of tumor ablation and cutaneous photosensitization associated with this photosensitizer remain undefined.

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In this study, we assessed the phototoxic effects of AlPCTS on membrane-bound epidermal cytochrome P-450 and associated enzyme activities *in vitro*. Our data indicate that addition of AlPCTS followed by irradiation to red light (~675 nm) destroys the heme-protein cytochrome P-450 and associated monooxygenase activities and that singlet oxygen plays a major role in this photocatalytic process.

MATERIALS AND METHODS

Chemicals NADPH, 2,5-dimethylfuran (2,5-DMF), sodium benzoate, mannitol, superoxide dismutase (SOD), catalase, and β-carotene were obtained from Sigma Chemical Co, St. Louis, MO. AlPCTS was a product of Porphyrin Products, Logan, UT. All other chemicals were of the highest purity commercially available.

Preparation of Tissue Four-day-old Sprague-Dawley rats were killed by decapitation, and whole skin excised and immediately placed in ice-cold 0.15 M KCl. Each skin was placed epidermal-side-down on a covered glass Petri dish containing crushed ice and scraped with a sharp scalpel blade to remove subcutaneous fat and muscle. Epidermis was separated from whole skin, minced and homogenized for preparation of microsomes as described previously (15). The epidermal microsomal pellet was washed once using 0.1M phosphate buffer (pH 7.4) containing 10^{-4}M $MgCl_2$, 20% glycerol, 10mM dithiothreitol and EDTA, and was homogenized and suspended in the same buffer. The final microsomal suspension was brought to a concentration of 2 mg protein/ml. The protein concentration was determined by the method of Bradford (16) using bovine serum albumin as standard.

Irradiation Incubation mixtures containing 0.1 M phosphate buffer (pH 7.4), epidermal microsomal protein (2 mg/ml), AlPCTS (0.5 to 2.0 μg/ml), in a final volume of 1 ml were irradiated under a metal halide lamp equipped with a red filter. The emission was monitored using an IL-700 Research Radiometer (International Light, Newbury-Port, MA) and found to be ~675 nm. The incubation mixture was placed in a Petri dish surrounded by crushed ice and exposed to ~675 nm radiation for desired doses of radiant energy. All quenchers were dissolved in 0.1 M phosphate buffer (pH 7.4).

Gel Electrophoresis This was done by the procedure of Laemmli (17) using 12.5% acrylamide gels in the presence of 0.1% sodium dodecyl sulfate (SDS) and 0.1% 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue.

Enzyme Assays Aryl hydrocarbon hydroxylase (AHH) activity was determined by a modification of the method of Nebert and Gelboin (18) as described previously (19). The quantitation of phenolic metabolites was based on comparison of fluorescence to a standard solution of 3-hydroxybenzo(a)pyrene. 7-Ethoxycoumarin O-deethylase (ECD) activity was determined according to a slight modification of the procedure of Greenlee and Poland (20), the details of which were described by Bickers et al (15). 7-Ethoxyresorufin O-deethylase (ERD) activity was determined according to the procedure of Pohl and Fouts (21). The reaction was initiated by the addition of 1.5 μM 7-ethoxyresorufin in 5 μl DMSO and was incubated at 37°C for 30 min in a Dubnoff metabolic shaker. The reaction was

terminated by the addition of 2.0 ml of methanol. Fluorescence of the deethylated metabolite was measured at excitation and emission wavelengths of 550 nm and 585 nm, respectively. The quantitation of the deethylated metabolite was based on comparison of fluorescence of standard resorufin. The effect of various quenchers was assessed by adding them to the reaction mixtures immediately prior to irradiation.

RESULTS

Photodestruction of AlPCTS-mediated cytochrome P-450 in epidermal microsomes. As shown in Figure 1, *in vitro* addition of AlPCTS to the epidermal microsomal suspension followed by ~675 nm irradiation resulted in a rapid and significant destruction of cytochrome P-450 and other proteins. The gel shows that the bands present in microsomes exposed to light alone or AlPCTS alone were virtually identical to those observed with control microsomes without light or AlPCTS. However, when microsomes containing AlPCTS were exposed to ~675 nm radiations, bands in the P-450 regions (~50 kd) disappeared. This effect was found to be dependent on both the dose of light, and on the concentration of AlPCTS.

Photodestruction of AlPCTS-mediated monooxygenase activities in epidermal microsomes. The data shown in Fig. 2 indicate that P-450 dependent monooxygenase activities such as AHH, ECD and ERD were also significantly diminished by the addition of AlPCTS and

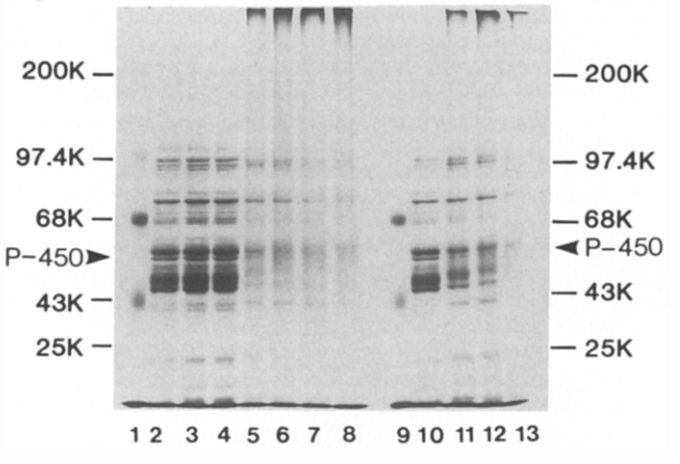

Fig.1 **SDS-polyacrylamide gel electrophoresis of epidermal microsomes containing AlPCTS and exposed to ~675 nm radiation.** Lanes 1 and 9, molecular weight standards; lanes 2 and 10, microsomes without AlPCTS and light; lanes 3 and 4, microsomes with either AlPCTS (1.0 μg/ml) or light (25J/cm^2) alone respectively; lanes 5-8, microsomes with 0.5, 1.0, 1.5 or 2.0 μg/ml AlPCTS respectively and exposed to ~675 nm radiation (25J/cm^2); lanes 11-13, microsomes with AlPCTS (1.0 μg/ml) and exposed to ~675 nm radiation for 10, 20 or 30J/cm^2 light fluence. In each lane 50 μg protein was loaded.

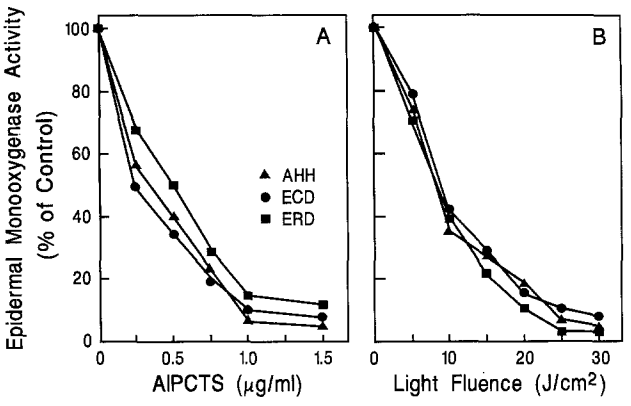

Fig.2 **AlPCTS-mediated photodestruction of epidermal monooxygenase activities. Panel A:** Various concentrations of AlPCTS were added to microsomes (2.0 mg protein/ml) and samples were exposed to ~675 nm radiation ($25J/cm^2$). **Panel B:** Microsomes containing AlPCTS (1.0 μg/ml) were exposed to ~675 nm radiation at varying light fluences. AHH, ECD, and ERD activities were determined in these samples as described in Materials and Methods, and found to be 1.29 ± 0.17 pmol 3-OH-BP/min/mg protein, 1.07 ± 0.12 pmol 7-OH coumarin/min/mg protein, and 0.73 ± 0.09 pmol resorufin/min/mg protein in the unirradiated control samples.

~675 nm irradiation. The destruction of these activities was more than 85% by the addition of AlPCTS (1.0 μg/ml) followed by ~675 nm radiation ($25J/cm^2$). The photodestruction of AHH, ECD and ERD activities was also found to be dependent on the concentration of AlPCTS (Fig. 2, panel A) and upon the dose of irradiation (Fig. 2, panel B).

Role of quenchers of singlet oxygen on AlPCTS-mediated photodestruction of epidermal monooxygenase activities. Several studies have clearly shown that photosensitizers in the presence of light generate singlet oxygen and other reactive species (22-25). Using several known quenchers of singlet oxygen (26), we assessed its involvement in AlPCTS-mediated photodestruction of epidermal microsomal monooxygenase activities. As shown by the data in Table 1, the quenchers of singlet oxygen significantly (64 to 80%) prevented the AlPCTS-mediated photodestruction of AHH, ECD and ERD activities. The protective effect of 2,5-DMF, histidine, β-carotene, and sodium azide was found to be comparable for all three enzyme activities. The effect of each of these quenchers was concentration dependent, however, in Table 1, the data are shown with maximum protective effect.

Role of quenchers of superoxide anion and hydrogen peroxide in AlPCTS-mediated photodestruction of epidermal monooxygenase activities. In addition to singlet oxygen we also evaluated the possible involvement of superoxide anion and hydrogen peroxide in

Table 1. Effect of Scavengers of Reactive Oxygen Species on AlPCTS-mediated photodestruction of Epidermal Microsomal Monooxygenase Activities

Incubation System[a]	Monooxygenase Activity[b]					
	AHH (pmol 3-OH-BP/min/mg protein)	% Protection	ECD (pmol 7-OH coumarin/min/mg protein)	% Protection	ERD (pmol resorufin/min/mg protein)	% Protection
Control	1.29 ± 0.17	-	1.07 ± 0.12	-	0.73 ± 0.09	-
Control + hv	1.32 ± 0.19	-	1.05 ± 0.15	-	0.78 ± 0.10	-
Control + AlPCTS	1.27 ± 0.13	-	1.03 ± 0.14	-	0.70 ± 0.08	-
Complete	0.12 ± 0.01[c]	-	0.07 ± 0.00[c]	-	0.06 ± 0.01[c]	-
+ histidine (10 mM)	0.83 ± 0.11	64[d]	0.75 ± 0.09	70[d]	0.48 ± 0.06	66[d]
+ 2,5-DMF (10 mM)	0.91 ± 0.16	71[d]	0.80 ± 0.07	75[d]	0.53 ± 0.03	72[d]
+ β-carotene (10 mM)	0.89 ± 0.15	69[d]	0.73 ± 0.06	68[d]	0.52 ± 0.04	71[d]
+ sodium azide (10 mM)	1.03 ± 0.13	80[d]	0.85 ± 0.08	79[d]	0.57 ± 0.07	78[d]
+ SOD (200 ul/ml)	0.11 ± 0.02	NE[e]	0.07 ± 0.01	NE	0.05 ± 0.01	NE
+ catalase (200 ul/ml)	0.13 ± 0.03	NE	0.09 ± 0.02	NE	0.07 ± 0.02	NE
+ sodium benzoate (10 mM)	0.12 ± 0.01	NE	0.08 ± 0.02	NE	0.06 ± 0.01	NE
+ mannitol (10 mM)	0.13 ± 0.03	NE	0.06 ± 0.01	NE	0.06 ± 0.01	NE
+ ethanol (10 mM)	0.12 ± 0.04	NE	0.07 ± 0.02	NE	0.06 ± 0.01	NE

[a] Complete = Epidermal microsomes (2.0 mg protein/ml) in phosphate buffer were incubated with AlPCTS (1.0 μg/ml) and exposed to ~675nm light (25J/cm^2).
[b] Data represent mean ± S.E. of 6 individual values.
[c] Significantly different when compared to control ($p<0.005$).
[d] Significantly different when compared to complete ($p<0.005$).
[e] No effect.

the AlPCTS-mediated photodestruction of epidermal microsomal monooxygenase activities. As shown in Table 1, the addition of SOD, a quencher of superoxide anion, or catalase, a quencher of hydrogen peroxide, even up to 200 μg/ml showed no protection against the AlPCTS-mediated photodestruction of monooxygenase activities, suggesting that superoxide anion and hydrogen peroxide are not directly involved in this photodestructive process.

Role of quenchers of hydroxyl radical on AlPCTS-mediated photodestruction of epidermal monooxygenase activities. The role of the hydroxyl radical in the photodestruction of monooxygenase activities was studied by using various concentrations of sodium benzoate, mannitol, and ethanol. The data shown in Table 1 indicate that even up to 10 mM concentration, these scavengers did not afford protection against the AlPCTS-mediated photodestruction of AHH, ECD and ERD activities. These data suggest that like superoxide anion and hydrogen peroxide, the hydroxyl radical also does not play an important role in the AlPCTS-mediated photodestruction of monooxygenase activities.

DISCUSSION

The vulnerability of cellular membranes including neutrophil plasma membranes (23), mammary mitochondrial membranes (27), and egg phosphatidyl-choline liposomal membranes (24) to photosensitizer-induced photodamage is well known. In this study, we clearly demonstrate that membrane-bound epidermal microsomal cytochrome P-450 is also highly vulnerable to photocatalytic destruction by AlPCTS and light, and that singlet oxygen plays a major role in this photosensitization process. Since the photodynamic destruction of cytochrome P-450 associated monooxygenase activities has an absolute requirement for oxygen, as observed by using various quenchers of singlet oxygen species, it is reasonable to suggest that singlet oxygen is directly involved in this destruction process.

Several forms of reactive oxygen species are known to exist including singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical. These represent sequential reduction of molecular oxygen and are interconvertible under certain conditions. Therefore, it is often difficult, if not impossible, to assign a specific toxic effect to one or another species of reactive oxygen in biological systems. However, our results clearly indicate that singlet oxygen is a major oxidant in the photodestruction of microsomal membranes by AlPCTS and light. Various compounds known

to diminish the reactivity of singlet oxygen such as 2,5-DMF, histidine, β-carotene, and sodium azide, each afforded substantial protection against the destructive effect of AlPCTS and light on cytochrome P-450 dependent monooxygenase activities.

In summary, our results indicate that singlet oxygen is a major species of activated oxygen involved in AlPCTS-mediated photodestruction of cytochrome P-450 and associated monooxygenases, and since singlet oxygen is generated as a result of several complex biological processes (28,29), it is highly likely that this moiety plays a major role in AlPCTS photosensitization. These results suggest that microsomal membranes contain moieties which are uniquely susceptible to AlPCTS photosensitization which can damage the cytochrome P-450 system, thereby resulting in the loss of an enzyme system responsible for the detoxification of drugs and chemicals. Whether such an effect on this enzyme system participates in this process of tumor necrosis evoked by PDT remains to be developed.

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REFERENCES

1. Gomer, C.J., Rucker, N., Ferrario, A., and Wang, S. (1989) Radiation Res., 120, 1-8.
2. Dougherty, T.J. (1984) CRC Crit. Rev., 2, 83-116.
3. Dougherty, T.J. (1987) Photochem. Photobiol., 45, 879-889.
4. Riesz, P. and Krishna, C.M. (1987) Proc. Soc. Photo-Optic Instrum. Eng., 847, 15-28.
5. Tralau, C.J., Young, A.R., Walker, N.P.J., Vernon, D.I., MacRobert, A.J., Brown, S.B., and Bown, S.G. (1989) Photochem. Photobiol., 49, 305-312.
6. Bown, S.G., Tralau, C.J., Coleridge Smith, P.D., Akdemir, D. and Wieman, T.J. (1986) Br. J. Cancer, 54, 43-52.
7. Chan W-S., Marshall, J.F. and Hart, I.R. (1987) Photochem. Photobiol., 46, 867-871.
8. Tralau, C.J., Barr, H., Sandeman, D.R., Barton, T., Lewin, M.R. and Bown, S.G. (1987) Photochem. Photobiol., 46, 777-781.
9. Tralau, C.J., MacRobert, A.J., Coleridge-Smith, P.D., Barr, H. and Bown, S.G. (1987) Br. J. Cancer, 55, 389-395.
10. Roberts, W.G., Smith, K.M., McCullough, J.L. and Berns, M.W. (1989) Photochem. Photobiol., 49, 431-438.
11. Chan, W-S., Marshall, J.F., Lam, G.Y.F. and Hart, I.R. (1988) Cancer Res., 48, 3040-3044.
12. Spikes, J.D. (1986) Photochem. Phtobiol., 43, 691-699.

13. Barr, H., Tralau, C.J., MacRobert, A.J., Krasner, N., Boulos, P.B., Clark, C.G. and Bown, S.G. (1987) Br. J. Cancer, 56, 111-118.
14. Ben-Hur, E. (1990) The Spectrum 3, 3-4.
15. Bickers, D.R., Dixit, R. and Mukhtar, H. (1982) Biochem. Biophys. Res. Commun., 108, 1032-1039.
16. Bradford, M.M. (1976) Anal. Biochem., 72, 248-254.
17. Laemmli U.K. (1970) Nature 227, 680-685.
18. Nebert, D.W. and Gelboin, H.V. (1964) J. Biol. Chem., 243, 2370-2385.
19. Mukhtar, H. and Bickers, D.R. (1981) Drug Metab. Dispos., 9, 311-314.
20. Greenlee, W.F. and Poland, A. (1978) J. Phamracol. Exp. Ther., 205, 596-605.
21. Pohl, R.J., and Fouts, J.R. (1980) Anal. Biochem., 107, 150-155.
22. Evensen, J.F. and Moan, J. (1982) B. J. Cancer, 45, 456-465.
23. Girotti, A.W. (1983) Photochem. Photobiol., 38, 745-751.
24. Goyal, G.C., Blum, A. and Grossweiner, L.I. (1983) Cancer Res., 43, 58216-5830.
25. Spikes, J.D. (1975) Ann. NY Acad. Sci., 244, 496-508.
26. Chan, W.S., Svensen, R., Phillips, D. and Hart, I.R. (1986) Br. J. Cancer, 53, 255-264.
27. Gibson, S.L., and Hilf, R. (1983) Cancer Res., 43, 4191-4197.
28. Bodaness, R.S., P.C. Chan (1977) J. Biol. Chem., 252, 8554-8560.
29. Kenlogg, E.W. and Fridovich, I. (1975) J. Biol. Chem., 250, 8812-8817.